[1] Mass Spectrometric Quantification of F_2-Isoprostanes in Biological Fluids and Tissues as Measure of Oxidant Stress

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Introduction

Free radicals derived primarily from oxygen have been implicated in the pathophysiology of a wide variety of human disease including atherosclerosis, cancer, neurodegenerative disorders, and even the normal aging process.[1] Much of the evidence for this, however, is indirect or circumstantial, largely because of limitations of methods that are available to quantify free radicals or the products they produce in biological systems. This is a particular problem when noninvasive approaches are used to assess oxidant injury in animals or humans.[2]

Measures of lipid peroxidation are frequently utilized to implicate free radicals in pathophysiological processes. These measurements include quantification of short chain alkanes, malondialdehyde, or conjugated dienes. Each of these, however, suffers from problems related to specificity and sensitivity, especially when utilized to quantify oxidant stress *in vivo*. Further, artifactual generation of these lipid peroxidation products can occur *ex vivo* and factors such as endogenous metabolism can affect levels of compounds measured.[2]

In 1990, we reported that a series of prostaglandin(PG) F_2-like compounds, termed F_2-isoprostanes (F_2-IsoPs), are produced *in vivo* in humans by a non-cyclooxygenase free radical-catalyzed mechanism involving the peroxidation of arachidonic acid.[3] Formation of these compounds initially involves the generation of four positional peroxyl radical isomers of arachidonic acid which undergo endocyclization to PGG_2-like compounds that are subsequently reduced to PGF_2-like compounds. Four F_2-IsoP regioisomers are formed, each of which can theoretically comprise eight racemic diastereomers. In support of the proposed mechanism of formation, we

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have obtained direct evidence both *in vitro* and *in vivo* that each of the four classes of regioisomers are formed.[4]

We have accumulated a large body of evidence that suggests that quantification of F_2-IsoPs represents a reliable and useful approach to assess lipid peroxidation and oxidant stress *in vivo*. First, the formation of F_2-IsoPs has been shown to increase dramatically in various animal models of free radical injury, and these increases correlate with measures of tissue damage. For example, circulating concentrations of F_2-IsoPs increase up to 100-fold following the administration of diquat to selenium-deficient rats or CCl_4 to normal rats.[3] These studies have also illustrated that quantification of F_2-IsoPs provides a much more sensitive and accurate method to assess lipid peroxidation *in vivo* compared with other markers. As an example, following administration of CCl_4 to rats, levels of F_2-IsoPs esterifed to hepatic lipids increased greater than 80-fold, whereas levels of malondialdehyde (MDA) increased only 2.7-fold.[5] In another study, we found that measuring F_2-IsoP afforded a significantly more sensitive indicator of CCl_4-induced lipid peroxidation compared with measurement of lipid hydroperoxides by mass spectrometry.[6] Further, concentrations of F_2-IsoPs are present that are easily detected in normal human biological fluids such as plasma and urine.[3] This allows the definition of a normal range which permits an assessment of small increases in the formation of F_2-IsoPs in settings of mild oxidant stress. In addition, F_2-IsoPs can be detected in virtually every type of biological fluid analyzed thus far, including plasma, urine, cerebrospinal fluid, bile, lymph, bronchoalveolar lavage fluid, and synovial fluid.[7] Levels of these compounds are also detectable in all types of tissues examined to date. These include liver, kidney, stomach, brain, lung, vascular tissue, muscle, and heart. Thus, the fact that F_2-IsoPs are detectable in various tissues and fluids provides the opportunity to assess the formation of these compounds at local sites of oxidant injury. Finally, we have shown that F_2-IsoPs are increased in a number of human disorders associated with enhanced oxidant stress, including chronic heavy cigarette smoking, atherosclerosis, hepatorenal syndrome, and systemic sclerosis.[7]

The precursor of the F_2-IsoPs is arachidonic acid. The vast majority of arachidonic acid present *in vivo* exists esterified to phospholipids. Previously, we reported the novel finding that F_2-IsoPs are initially formed *in*

[4] R. J. Waugh, J. D. Morrow, L. J. Roberts, and R. C. Murphy, *Free Rad. Biol. Med.* **23,** 943 (1997).

[5] A. W. Longmire, L. L. Swift, L. J. Roberts, J. A. Awad, R. F. Burk, and J. D. Morrow, *Biochem. Pharm.* **47,** 1173 (1994).

[6] W. R. Matthews, R. McKenna, D. M. Guido, T. W. Petry, R. A. Jolly, J. D. Morrow, and L. J. Roberts, *Proc. 41st A.S.M.S. Conf. Mass Spectrometry,* 865 (1993).

[7] J. D. Morrow and L. J. Roberts, *Prog. Lipid Res.* **36,** 1 (1997).

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situ from arachidonic acid esterified to phospholipids and then subsequently released in the free form by phospholipases.[8] This observation provides the basis for an important concept regarding the assessment of isoprostane formation *in vivo* in that when isoprostane formation is quantified, total production of F_2-IsoPs may be more accurately assessed by measuring levels of both free and esterified compounds. The relative ratio of esterified and free compounds presumably would depend on the degree of activation of phospholipases in settings of oxidant injury.[5] For example, following administration of CCl_4 to rats, F_2-IsoP esterified to liver phospholipids increases rapidly with levels maximal at 2 hours, while increases in circulating levels of free F_2-IsoPs are delayed and maximal at 8 hours.[7] On the other hand, when quantified in tissues, the majority of F_2-IsoPs are esterified in phospholipids, and thus, analyzing levels of esterified compounds, as opposed to both free and esterified F_2-IsoPs, appears to provide an accurate assessment of oxidant injury in specific organs.[7]

The purpose of this chapter is to detail methods employed for the analysis of F_2-IsoPs from biological sources utilizing mass spectrometric techniques. Specific examples are given demonstrating both the utility and limitations of the assay. Procedures will be outlined for the analysis of both free F_2-IsoPs and F_2-IsoPs esterified to phospholipids. F_2-IsoPs from biological sources can only be quantified as free compounds using gas chromatography (GC)/mass spectrometry (MS). Thus, to measure levels of these compounds esterified to phospholipids, the phospholipids are first extracted from the tissue sample and subjected to alkaline hydrolysis to release free F_2-IsoPs. Free F_2-IsoPs are then quantified using the same procedure for the measurement of free compounds in biological fluids. The following methods, therefore, first outline the extraction and hydrolysis of F_2-IsoPs from tissue lipids. Subsequently, the method of analysis for free compounds is discussed.

Handling and Storage of Biological Fluids and Tissues for Quantification of F_2-Isoprostanes

As discussed, F_2-IsoPs have been detected in all biological fluids and tissues examined thus far. A potential drawback to measuring F_2-IsoPs as an index of endogenous lipid peroxidation is that they can be readily generated *ex vivo* in biological fluids such as plasma in which arachidonyl-containing lipids are present. This occurs not only when biological fluids or tissues are left at room temperature, but also when they are stored at

[8] J. D. Morrow, J. A. Awad, H. J. Boss, I. A. Blair, and L. J. Roberts, *Proc. Natl. Acad. Sci. USA* 89, 10721 (1992).

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−20°C.[7] However, we have found that the formation of F_2-IsoPs does not occur if biological fluids or tissues are processed immediately after procurement and if agents including butylated hydroxytoluene (BHT, a free radical scavenger) and/or triphenylphosphine (a reducing agent) are added to the organic solvents during extraction and hydrolysis of phospholipids.[7,8] Thus, samples to be analyzed for F_2-IsoPs should either be processed immediately or stored at −70°C. Ideally, samples should be rapidly frozen in liquid nitrogen prior to placement at −70°C. This is especially important for tissue samples, and we routinely snap freeze them with a clamp prior to storage at −70°C since it is known that inner areas of tissue samples that are not snap frozen may remain in a liquid state for a period of time, even when placed at −70°C.

Extraction and Hydrolysis of F_2-Isoprostane-Containing Phospholipids in Tissues and Biological Fluids

To 0.05–1 g of tissue is added 20 ml of ice-cold Folch solution, $CHCl_3$/methanol (2:1, v/v), containing 0.005% BHT in a 40 ml glass centrifuge tube. As discussed, the presence of BHT during extraction and hydrolysis of lipids is important since it completely inhibits *ex vivo* formation of F_2-IsoPs during this procedure.[7] The tissue is then homogenized with a blade homogenizer (PTA 10S generator, Brinkmann Instruments, Westbury, NY) for 30 seconds and mixture allowed to stand, sealed under nitrogen at room temperature for 1 hour. Four milliliters of aqueous NaCl (0.9%) is then added and the solution is vortexed vigorously and centrifuged at 800*g* for 10 minutes. After centrifugation, the upper aqueous layer is discarded and the lower organic layer carefully separated from the intermediate semisolid proteinaceous layer.

The organic phase containing the extracted lipids is then transferred to a 100 ml conical bottom flask and evaporated under vacuum to dryness. Four milliliters of methanol containing BHT (0.005%) and 4 ml of aqueous KOH (15%) is then added to the residue and incubated at 37°C for 30 minutes to effect hydrolysis and release of the F_2-IsoPs. The mixture is then acidified to pH 3 with 1 *M* HCl and diluted to a final volume of 80 ml with pH 3 water in preparation for extraction of free F_2-IsoPs as discussed below. Dilution of the methanol in the solution with water to 5% or less is necessary to ensure proper column extraction of F_2-IsoPs in the subsequent purification procedure.

For the extraction of lipids containing esterified F_2-IsoPs in biological fluids such as plasma, as opposed to tissue, a different method is used.[7,9]

[9] J. D. Morrow, B. Frei, A. W. Longmire, J. M. Gaziano, S. M. Lynch, Y. Shyr, W. E. Strauss, J. A. Oates, and L. J. Roberts, *New Engl. J. Med.* **332**, 1198 (1995).

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We have found that the addition of BHT alone to the Folch solution used to extract plasma lipids does not suppress *ex vivo* lipid peroxidation entirely. On the other hand, the addition of both triphenylphosphine and BHT to the organic solvents used to extract plasma lipids entirely prevents autoxidation. Thus, to 1 ml of a biological fluid such as plasma in a 40 ml conical glass centrifuge tube is added 20 ml of ice-cold Folch solution, $CHCl_3$/methanol (2:1, v/v), containing 0.005% BHT and triphenylphosphine (5 mg). The mixture is shaken for 2 minutes and then 10 ml of 0.043% $MgCl_2$ in water is added. The mixture is shaken again for 2 minutes and then centrifuged at 800*g* for 10 minutes. The organic layer is removed and dried under nitrogen and the residue is then subjected to hydrolysis using the same procedure outlined above for tissue lipids.

Purification, Derivatization, and Quantification of F_2-Isoprostanes

Quantification of F_2-IsoPs by GC/negative-ion chemical ionization (NICI) MS is extremely sensitive with a lower limit of detection in the range of 1–5 pg using a deuterated internal standard with a $^2H_0$ blank of less than 5 parts per thousand. Thus, it is usually not necessary to assay more than 1–3 ml of a biological fluid or a lipid extract from more than 50–100 mg of tissue. Further, because urinary levels of F_2-IsoPs are high (typically greater than 1 ng/ml), we have found 0.2 ml of urine is more than adequate to quantify urinary F_2-IsoPs. The following assay procedure that is described and summarized in Fig. 1 is the method used for analysis of free F_2-IsoPs in plasma, but is equally adaptable to other biological fluids and hydrolyzed lipid extracts of tissues. Previously, we had reported an alternative method for the quantification of F_2-IsoPs in urine involving two thin-layer chromatographic steps,[10] but we have found that urinary F_2-IsoPs are accurately measured by the same assay utilized to quantify these compounds in plasma or other biological fluids.

Following acidification of 3 ml of plasma to pH 3 with 1 *M* HCl, 200–1000 pg of a deuterated standard is added. Previously, we had employed [$^2H_7$]9α,11β-PGF_2 or [$^2H_4$]$PGF_{2\alpha}$ as an internal standard. We had synthesized the former compound from deuterium-labeled arachidonic acid, and the latter compound is available commercially. We have, however, begun to use a deuterium-labeled isoprostane, [$^2H_4$]8-iso-$PGF_{2\alpha}$, as an internal standard. This compound is also available from commercial sources (Cayman Chemical, Ann Arbor, MI) and we have found it to be of equal accuracy compared to the other standards. After addition of the internal standard, the mixture is vortexed and applied to a C_{18} Sep-Pak column

[10] J. D. Morrow and L. J. Roberts, *Methods Enzymol.* **233**, 163 (1994).

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